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mRNA Expression of Variant Fas Molecules in Acute Leukemia Cells

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Fas (Apo-1/CD95) is a cell membrane receptor involved in apoptotic cell death. Soluble variant forms (sFas) lacking the transmembrane domain due to alternative splicing have been identified. Up-regulation of sFas expression is reportedly implicated in prereceptorial blockage of Fas-induced apoptosis in a dose-dependent manner. We examined mRNA expression of Fas and sFas in fresh leukemia cells. All leukemia cells expressed both mRNAs of full-length Fas (FasFull) and sFas with deletion of exon6 (FasDel6). The ratio of FasFull/FasDel6 mRNA expression was not always correlated with Fas-mediated growth inhibition. Interestingly, in a 6-year-old boy with acute myelogenous leukemia, blast cells obtained at onset and at the time of bone marrow relapses expressed distinct amounts of FasDel6 mRNA. Furthermore, the level of FasDel6 expression appeared to be correlated with Fas-resistance in leukemia blasts. In addition, sFas protein levels were elevated in patients' sera at onset with subsequent return to normal levels after complete remission. These results indicated that sFas could be synthesized and released by leukemia blasts and suggested that up-regulation of Fas variant transcript might render leukemia blasts resistant to Fas-mediated growth inhibition in certain cases. Am. J. Hematol. 62:150–158, 1999.

Key words: variant Fas molecules; apoptosis; acute leukemia

INTRODUCTION

The human Fas protein (Apo-1/CD95) is a cell-surface receptor, belonging to the nerve growth factor (NGF)/tumor necrosis factor (TNF) receptor superfamily, and is known to mediate apoptosis after interaction with the Fas ligand (FasL) or cross-linking with agonistic anti-Fas antibodies [1–3]. Fas is a 48-kDa Type I membrane glycoprotein composed of 3 domains; an extracellular domain comprising 3 cysteine-rich motif subdomains characteristic of the superfamily, a transmembrane domain, and a highly conserved intracellular domain known as a death domain [4]. The death domain binds Fas-associated death domain protein (FADD) [5] which links Fas to a cascade of interleukin-1β-converting enzyme (ICE)-like proteases known as caspases [6,7]. The Fas-based death pathway plays an important regulatory role for the elimination of cells in vivo. The physiological importance of Fas is evident from both murine and human studies showing that spontaneous loss of function mutations of Fas results in lymphoproliferative disorders and autoimmune disease [8,9]. Fas-mediated apoptosis also contributes to cytotoxic T-lymphocyte cytotoxicity [10–12].

Our previous studies demonstrated that Fas was expressed on majority of human leukemia cells, although the intensity of expression was variable [13]. The cross-linking with anti-Fas monoclonal antibody (MoAb) could induce apoptotic cell death in certain cases of leukemia. However, in half of Fas-expressing leukemia cases, cell growth was not inhibited with the treatment of anti-Fas MoAb. Thus the resistant phenotype in Fas-insensitive cells may involve mechanisms other than those relating to the presence of cell-surface Fas. The molecular mechanisms mediating Fas-resistance are complex and involve both post-receptorial and pre-receptorial events. Post-receptorial resistance mecha-

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nisms are known to include the high expression of Fas-associated phosphatase-1 (FAP-1) [14], expression of truncated Fas receptor lacking the intracytoplasmic signaling domain [15], over-expression of bcl-2 [16] and bcl-X_L proteins [17], and expression of caspase regulators such as FLICE-like inhibitory protein (FLIP) [18] and inhibitor of apoptosis proteins (IAP) [19]. Prereceptorial resistance can be mediated by the soluble Fas (sFas) protein, which antagonizes both anti-Fas antibody and FasL killing in a dose-dependent manner [20,21]. sFas was found to be elevated in the sera from patients with autoimmune diseases, B- and T-cell leukemias [22] and nonhematopoietic malignancies such as melanoma, breast cancer, and colon cancer [23]. Human phytohaemagglutinin (PHA)-activated lymphocytes and several tumor cell lines express, in addition to the full-length Fas mRNA, alternatively spliced mRNA variants coding for sFas proteins. To date, five mRNA variants have been described, although their clinical significance has not been identified [21].

In the present study, we have demonstrated that the Fas mRNA variants, mostly characterized by a deletion of the transmembrane exon 6 domain, are variably expressed in primary leukemia cells. The serum levels of sFas proteins were clearly elevated in patients at onset with subsequent return to normal levels after complete remission. In addition, the analysis of Fas mRNA expression in a 6-year-old boy with acute myelogenous leukemia (AML) suggested the correlation between the resistance to Fas-mediated cell death and expression of Fas mRNA variants.

MATERIALS AND METHODS

Leukemia Cells

Bone marrow (BM) samples were taken from 9 cases of acute lymphoblastic leukemia (ALL) and 7 cases of AML at onset. At the time of the study, none of the patients had received any treatment. In addition, BM samples were obtained from a patient with AML (case 1) at the time of the first and second hematological relapses. Nucleated BM cells were isolated by Ficoll-Hypaque density centrifugation. The cell suspension contained more than 95% leukemia cells as assessed by May–Giemsa morphology and immunophenotypic characterization. Every case of ALL (age range 3–11 years) and AML (age range 4–14 years) enrolled in this study was classified according to the criteria of the French–American–British (FAB) committee [24]. Leukemia cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (complete medium). MML-1 and MML-1R cells were established from a patient with AML-M1 in our laboratory and both cell lines were maintained in complete medium [25].

Peripheral blood was obtained from healthy donors. Mononuclear cells were isolated by Ficoll-Hypaque density centrifugation. The isolated mononuclear cells were resuspended in complete medium with 10 IU/ml of interleukin-2 (IL-2) and cultured at 37°C. They were then subcultured every 5 days in fresh complete medium containing 10 IU/ml of IL-2. After 14 days of culture, activated lymphocytes were harvested and used in subsequent experiments.

Patients' Sera

Samples of sera were obtained from three ALL patients (cases 2, 4, and 6) and four AML patients (cases 1, 3, 5, and 7) at onset when BM samples were taken. In addition, sera were collected from patients, except for two AML patients (cases 1 and 3) who did not achieve complete remission, after complete remission had been successfully obtained. An additional serum sample was also obtained from a nonresponsive AML patient (case 3) after initial induction chemotherapy had been performed. All samples were cryopreserved at −20°C until tested for sFas using ELISA.

Immunofluorescence Analysis

Flow cytometric analysis of Fas expression was performed as previously described [13]. In brief, 10^6 cells were incubated with 10 μg/ml fluorescein-isothiocyanate-conjugated anti-Fas MoAb (clone UB2, mouse IgG_1, Medical and Biological Laboratories, Nagoya, Japan) for 30 min at 4°C. After washing with phosphate-buffered saline (PBS) containing 0.08% sodium azide, the intensity of fluorescence was analyzed with a FACScan flow cytometer (Nippon Becton Dickinson, Tokyo, Japan). Isotype-matched control MoAb was used to determine nonspecific binding. The mean fluorescence intensity of control MoAb was invariably low with small standard deviation among all samples assayed, indicating that nonspecific binding of MoAb would be minimal. Data were expressed as relative fluorescence intensity (RFI = mean fluorescence intensity of cells stained with anti-Fas MoAb/mean fluorescence intensity of cells stained with control MoAb). In order to verify reproducibility and accuracy of the assay, MML-1 cells were used as a Fas-positive control. Flow cytometric analysis of Fas expression was performed on leukemia cells separated from patients and MML-1 cells in parallel.

mRNA Expression of Fas and Variant Fas Molecules

Total cellular RNA was isolated from leukemia blasts using RNAzol-B method (Cinna/Biotex Laboratories, Houston, TX), following manufacturer's instructions. Single-stranded cDNA was synthesized with random hexamer oligonucleotides as primer and amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using TaKaRa RNA LA PCR Kit (TaKaRa Biomedicals,

Tokyo, Japan). Primers for the amplification of human Fas were designed according to the published sequences [20]. Human Fas cDNA was amplified using a sense primer (5′-CACTTCGGAGGATTGCTCAACA-3′; nucleotide 170–191) upstream of the ATG initiation codon and an antisense primer (5′-TATGTTG-GCTCTTCAGCGCTA-3′; nucleotide 1316–1336) downstream of the TAG termination codon. The condition for PCR were 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C for 30 cycles. The efficiency of reverse transcription and the amount of RNA used in RT-PCR were verified by detection of the human β-actin mRNA (sense primer, 5′-ACTACCTCATGAAGATCCTCA-3′; antisense primer, 5′-CAGGAGGAGCAATGATCT-TGA-3′). PCR products were analyzed on a 3.5% acrylamide gel and visualized by ethidium bromide staining. The intensity of ethidium bromide staining of each band was analyzed by NIH Image software for band quantification (Yodosha, Tokyo, Japan).

For PCR-Southern blot analysis, RT-PCR products were separated by electrophoresis on 2% agarose gels, and blotted onto nylon membrane (Bio-Rad Laboratories, Hercules, CA) by the alkaline transfer method. The blots were hybridized with horseradish peroxidase-conjugated oligonucleotide probes specific for the transmembrane region (exon 6 probe, 5′-CTTGGGGTGGCTTT-GTCTTCTTCTTTTGCC-3′; nucleotide 713–742) and the extracytoplasmic region (exon 5 probe, 5′-CAAGGAATGCACACTCACCAGCAACACCAAG-3′; nucleotide 656–686) of the human Fas cDNA. The probes bound to blots were detected using ECL direct nucleic acid labeling and detection systems (Amersham Life Science, Buckinghamshire, U.K.).

Cell Proliferation Assay

Proliferation of leukemia blasts was evaluated by measuring [^{3}H]thymidine (TdR, 925 GBq/mM, Amersham Corp., Arlington Heights, IL) incorporation. In brief, blast cells isolated from patients' BM (2×10^5/100 μl) were incubated with agonistic anti-Fas MoAb (clone CH-11, mouse IgM, Medical and Biological Laboratories, Nagoya, Japan) at the concentration of 10 to 1000 ng/ml. After 48 h incubation, cells were pulsed with [^{3}H]TdR (37 kBq/well) for an additional 4 h, and [^{3}H]TdR uptakes were determined by liquid scintillation counting. All experiments were performed in triplicate and SD was always <10% of mean cpm. Pre-irradiated (30 Gy) cells were run as controls to assess the background [^{3}H]TdR incorporation, which was invariably <500 cpm. Data (mean cpm) obtained from cultures treated with anti-Fas MoAb were set against the control values with isotype-matched control MoAb. Results were expressed as percentage of inhibition (% inhibition), which was calculated according to the following equation:

$$\% \text{ inhibition} = [(\text{mean cpm in control culture} - \text{mean cpm in experimental culture})/(\text{mean cpm in control culture})] \times 100.$$

Colorimetric DNA-Fragmentation Assay

Apoptosis was measured by flow cytometry according to a previously published procedure with minor modifications [26,27]. Leukemia cells (2×10^6/ml) were cultured with isotype-matched control MoAb for 3 days to evaluate baseline DNA fragmentation. Cells were also cultured with agonistic anti-Fas MoAb (clone CH-11) at the concentration of 1000 ng/ml. In order to record DNA histogram, about 10^6 cells were pelleted, resuspended in 200 μl PBS, and fixed rapidly with 2 ml 70% ice-cold ethanol. Cells were centrifuged from the fixative, resuspended in 1 ml PBS containing 10 μg/ml PI, 100 μg/ml ribonuclease A, and incubated at 37°C for 30 min. The excitation wavelength in the cytometer was 488 nm and orange (FL-2, 585 nm) fluorescence was recorded with FACScan flow cytometer. The apoptotic cell nuclei (sub-G1 peak in the DNA fluorescence histogram) was calculated using LYSYS software (Nippon Becton Dickinson). Baseline DNA fragmentation was observed in less than 13% of leukemia cells in cultures with control MoAb. Data were expressed as the percentage of Δ sub-G1 fraction ([percentage of sub-G1 nuclei in cultures with anti-Fas MoAb] − [percentage of sub-G1 nuclei in cultures with control MoAb]). It has been revealed that the cells from sub-G1 peak yielded a DNA ladder typical of apoptosis.

Detection of sFas Proteins

A sandwich enzyme immunoassay (sFas ELISA kit, MBL, Nagoya Japan) was carried out to determine sFas levels in patients' sera. The sFas proteins bound to anti-Fas polyclonal antibodies specific to the first epitope of the intracytoplasmic domain (amino acids 305–319) of the Fas molecule, which was coated on the microplate well. A horseradish peroxidase-conjugated anti-Fas MoAb against the second epitope of the extracytoplasmic domain (amino acids 110–120) was bound to the sFas captured by the first antibody. After washing to remove unbound material, a peroxidase substrate solution was added to each well. The reaction was then terminated, and the absorbance was measured at 450 nm with the chromogen using a microplate reader. The concentration of sFas (ng/ml) was calibrated from a dose–response curve based on reference standards. Mean sFas serum level (±SD) of age-matched healthy controls (n = 41) shown in manufacturer's instruction was 0.93 ± 0.3 ng/ml.

Statistical Analysis

All experiments were performed at least three times and shown to be reproducible. Levels of statistical significance were determined using the Student's *t*-test.

TABLE I. Fas Expression and Fas-Mediated Growth Inhibition in ALL Patients*

Patient no.	Surface marker	Fas expression (RFI)	FasFull/FasDel6 mRNA ratio	Cell proliferation: With control MoAb (mean cpm) 1,000 ng/ml	Cell proliferation: With anti-Fas MoAb (% inhibition) 10 ng/ml	100 ng/ml	1000 ng/ml	Fas-mediated DNA fragmentation (% of Δ sub-G1 fraction)
1	B precursor	0.9	1.00	1,123	<10%	18.3%	10.6%	<5%
2	B precursor	1.64	2.75	10,821	<10%	<10%	<10%	<5%
3	B precursor	4.52	2.29	33,782	<10%	<10%	19.4%	5.4%
4	B precursor	5.6	6.21	63,359	<10%	11.3%	19.8%	ND[a]
5	B precursor	1.72	1.54	4,617	<10%	<10%	<10%	<5%
6	B precursor	1.28	4.72	1,883	<10%	<10%	<10%	<5%
7	B precursor	2.64	1.79	1,380	<10%	<10%	31.8%	12.8%
8	B precursor	0.97	0.46	5,358	<10%	<10%	<10%	<5%
9	T	1.35	0.41	54,875	<10%	<10%	41.8%	25.2%

*Flow cytometric analysis was performed to determine the expression of cell-surface Fas. The results are expressed as RFI (relative fluorescence intensity). Fas mRNA transcripts were analyzed by RT-PCR (30 cycles) and FasFull/FasDel6 mRNA ratio was measured using an image analyzer. Thymidine incorporation was determined in the presence of anti-Fas MoAb as the measure of cell proliferation. Results were compared with the values in cell culture with isotype-matched control MoAb. Significant inhibition of thymidine uptake is indicated by squares ($P < 0.05$). Colorimetric DNA-fragmentation assay was performed to determine the fraction of apoptotic cell nuclei (sub-G1 fraction).
[a]ND, not determined.

RESULTS

Fas (CD95) Expression

We first analyzed Fas expression on ALL and AML cells by flow cytometric immunofluorescence analysis as shown in Tables I and II. The values of RFI were variable among tested samples (range: 0.9–5.6). The histograms from the flow cytometry data indicated that a relatively low RFI was not due to bright expression of Fas by a minor population, but due to dim expression of Fas by all cells (data not shown). ALL cells from two patients (cases 1 and 8) did not express Fas protein on the cell surface. On the other hand, AML cells were all positive for Fas. The RT-PCR analysis revealed that all ALL and AML samples expressed Fas mRNA, including two ALL patients (cases 1 and 8) which expressed no detectable cell-surface Fas protein (Fig. 1). Thus, Fas protein content estimated by flow cytometry did not meet the results of the RT-PCR in these two cases, although it is well known that mRNA level may not always reflect the protein expression status of the cells.

Fas-Mediated Growth Inhibition

To investigate the effect of anti-Fas MoAb on proliferation of leukemia blasts, cells were cultured for 48 h to achieve maximal stimulation and DNA synthesis was measured in the presence of anti-Fas MoAb. A significant inhibition of DNA synthesis was observed in ALL cells from 2 of 9 cases (Table I) and AML cells from 7 of 9 cases (Table II), when cells were cultured with anti-Fas MoAb to achieve concentrations as low as 100–1000 ng/ml. The suppression of DNA synthesis by anti-Fas MoAb was dose-dependent. The expression of Fas on AML cells (cases 1-1, 5, 6, and 7) used in this study and the cell proliferation data in the presence of anti-Fas MoAb have been partly described in our previous report [13]. In Fas-negative ALL cells from two patients (cases 1 and 8), DNA synthesis was not inhibited with the treatment of anti-Fas MoAb. To clarify whether Fas-mediated proliferation inhibition in leukemia cells is associated with DNA fragmentation, the most characteristic biochemical feature in apoptosis, leukemia cells treated with anti-Fas MoAb were analyzed with colorimetric DNA-fragmentation assay (Tables I and II). We found that the inhibition of DNA synthesis was accompanied by the increase of apoptotic nuclei in sub-G1 fraction, which suggested the induction of DNA fragmentation. In addition, we successfully established two leukemia cell lines, MML-1 and MML-1R from the same patient with AML [25]. The phenotype and characteristics of these cell lines have been stable for 2 years since their establishment. No distinct difference was identified in the intensity of Fas expression between MML-1 and MML-1R cells (RFI = 3.5 and 3.4, respectively). When MML-1 cells were treated with anti-Fas MoAb (1000 ng/ml) for 24 h, the number of apoptotic cells in sub-G1 fraction significantly increased up to 87.5%. However, MML-1R cells were completely resistant to Fas-mediated apoptotic cell death (sub-G1 fraction <5%). Taken together, it is concluded that there existed no apparent correlation between the sensitivity to Fas-mediated growth inhibition and the relative intensity of Fas expression on the cell surface, as consistently demonstrated in previous reports [13,28]. Thus, the failure of anti-Fas MoAb to trigger apoptotic cell death in insensitive leukemia cells may involve mechanisms other than those relating to the presence of Fas.

Expression of Fas and Variant Fas mRNA

To investigate the possibility that soluble forms of Fas molecules are expressed in human leukemia cells,

TABLE II. Fas Expression and Fas-Mediated Growth Inhibition in AML Patients*

Patient no.	FAB classification	Fas expression (RFI)	FasFull/FasDel6 mRNA ratio	Cell proliferation: With control MoAb (mean cpm) 1,000 ng/ml	Cell proliferation: With anti-Fas MoAb (% inhibition) 10 ng/ml	100 ng/ml	1000 ng/ml	Fas-mediated DNA fragmentation (% of Δ sub-G1 fraction)
1-1 (onset)	M1	1.76	2.42	10,677	<10%	<10%	27.9%	17.3%
1-2 (1st relapse)	M1	1.53	1.10	2,623	<10%	<10%	<10%	<5%
1-3 (2nd relapse)	M1	2.24	6.50	2,286	<10%	<10%	45.3%	27.7%
2	M1	3.06	2.60	143,745	<10%	<10%	52.0%	ND[a]
3	M1	4.58	6.48	4,391	13.3%	19.5%	23.9%	15.2%
4	M2	1.32	0.37	28,976	<10%	<10%	24.9%	7.8%
5	M3	4.95	2.01	53,085	<10%	46.4%	57.4%	40.1%
6	M4	2.54	3.13	10,840	13.2%	22.1%	23.1%	12.3%
7	M4	7.98	2.81	7,324	<10%	<10%	<10%	<5%

*See footnote to Table I.
[a]ND, not determined.

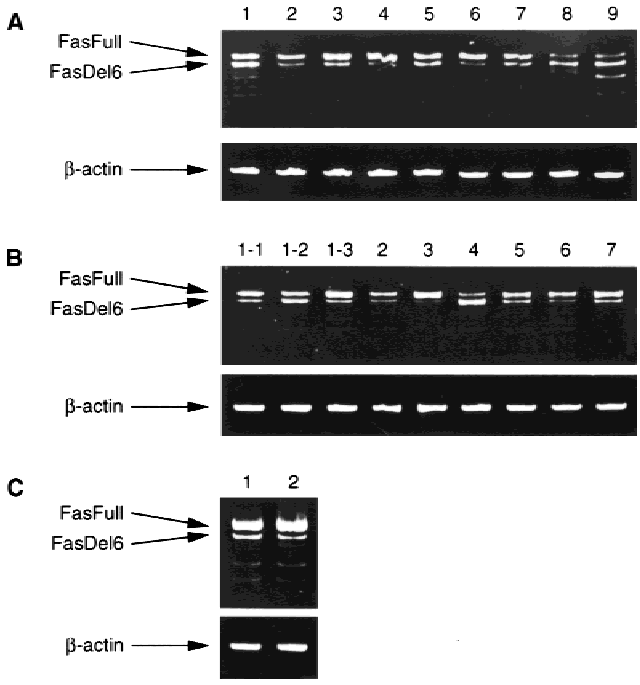

Fig. 1. RT-PCR analysis of FasFull and FasDel6 transcripts. The human Fas cDNA was amplified by RT-PCR (30 cycles) using appropriate primers representative of the 5′ or 3′ noncoding regions of the human Fas gene. (A) BM samples from 9 patients with ALL at onset (cases 1–9), (B) BM samples from a 6-year-old boy with AML (case 1-1, at onset; case 1-2, 1st BM relapse; case 1-3, 2nd BM relapse) and additional 6 patients with AML at onset (cases 2–7), (C) normal fresh lymphocytes (sample 1) and IL-2-activated lymphocytes (sample 2). The resultant PCR products were analyzed on a 3.5% acrylamide and visualized by ethidium bromide staining. Two predominant Fas transcripts (FasFull and FasDel6) were identified in all samples.

mRNA transcripts were analyzed by RT-PCR. In addition to the expected fragment (1167 bp) corresponding to the full-length cDNA named FasFull, a distinct smaller RT-PCR product (approximately 1100 bp) was identified in all 18 ALL and AML samples (Fig. 1). To characterize the smaller RT-PCR product, a series of PCR-Southern blot analysis was performed using oligonucleotide probes specific for exon 5 coding for the extracytoplasmic domains and exon 6 coding for the transmembrane domain (Fig. 2). The probe specific for exon 5 region was bound to both the FasFull fragment and the smaller cDNA fragment. However, the probe for exon 6 region could not react to the smaller 1100 bp cDNA fragment but could react to the 1167 bp FasFull fragment. The exon 6 probe used in this study was a oligonucleotide fragment corresponding to the transmembrane region between nucleotide position 700 and 762. Therefore, the smaller 1100 bp cDNA fragment named FasDel6 is most likely characterized by a deletion of approximately 60 bp corresponding to the transmembrane exon 6 of the human Fas cDNA. It is of note that the longer exposure of Southern blots hybridized with the exon 5 probe could allow us to identify four additional fainter bands (approximately 1060, 1000, 920, and 860 bp) in the majority of samples analyzed. The probe for exon 6 region could not react to these smaller fragments.

The ratio of FasFull to FasDel6 (FasFull/FasDel6) was examined using an image analyzer in order to clarify

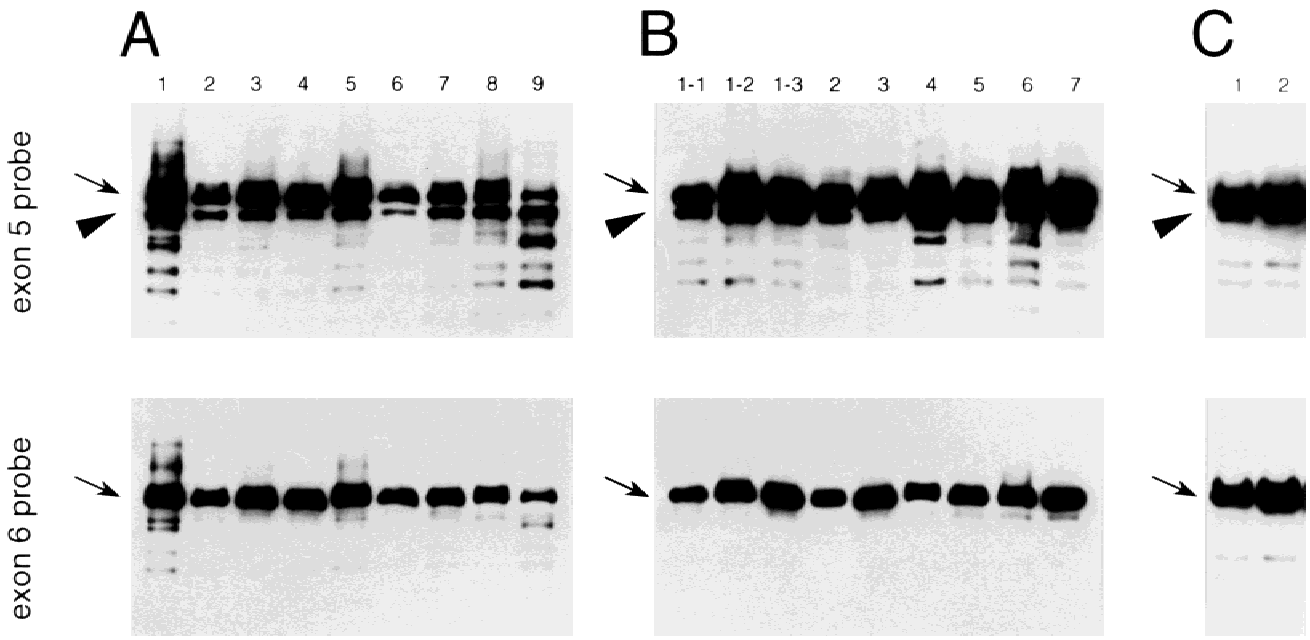

Fig. 2. PCR-Southern blot analysis of full-length Fas and Fas splicing variants. The RT-PCR products amplified after 30 PCR cycles were separated on 2% agarose gels and blotted onto nylon membrane. The blots were hybridized with oligonucleotide probes specific for the extracytoplasmic region (exon 5) or the transmembrane region (exon 6). (A) BM samples from 9 patients with ALL at onset (cases 1–9), (B) BM samples from a 6-year-old boy with AML (case 1-1, at onset; case 1-2, 1st BM relapse; case 1-3, 2nd BM relapse) and additional 6 patients with AML at onset (cases 2–7), (C) normal fresh lymphocytes (sample 1) and IL-2-activated lymphocytes (sample 2). FasFull and FasDel6 transcripts were indicated by arrows and arrowheads, respectively.

whether there was a discrepancy between the full-length Fas mRNA level and the variant Fas mRNA level in ALL and AML samples. First, to demonstrate that the full-length and variant Fas mRNA transcripts are produced with similar efficiency, PCR products from leukemia cells (AML case 5) after 20, 25, 30, 35, or 40 cycles were analyzed. Their RT-PCR reactions appeared to be linear up to 35 PCR cycles. When the band density was examined using an image analyzer, the mean FasFull/FasDel6 ratios were quite constant at 25, 30, 35, and 40 PCR cycles (2.1, 1.8, 2.0, and 1.9, respectively), indicating that FasFull and FasDel6 fragments were produced with similar efficiency. Freshly isolated lymphocytes and IL-2-activated lymphocytes were used as inter-assay standards (FasFull/FasDel6 ratios 3.74 and 5.60, respectively). As shown in Tables I and II, the ratios were quite variable between leukemia samples (range 0.37–6.48). There was no apparent correlation between the FasFull/FasDel6 ratio and the cellular lineage of leukemia blasts.

We further evaluated whether the FasFull/FasDel6 ratio and sensitivity to Fas-mediated growth inhibition might be correlated to each other. No significant correlation was, however, found in ALL or AML patient group at onset. In a 6-year-old boy with refractory AML, leukemia blasts obtained at onset (case 1-1) and at the time of the second BM relapse (case 1-3) were sensitive to Fas-mediated growth inhibition and showed relatively high FasFull/FasDel6 ratios (2.42 and 6.50, respectively), while BM blasts at the first BM relapse (case 1–2) were completely resistant to Fas-mediated growth inhibition and showed the lower FasFull/FasDel6 ratio (1.10) indicating the relative increase of FasDel6 transcript. The intensity of cell-surface Fas expression was comparable among these three samples. In addition, DNA synthesis of leukemia blasts isolated from this patient were invariably suppressed in the presence of 1000 U/ml TNF-α. The percent of growth inhibition assessed by thymidine incorporation was 74.4% at onset, 68.5% at the first BM relapse, and 86.6% at the second BM relapse. Moreover, mRNA of Fas-associating protein with death domain (FADD), receptor-interacting protein (RIP), and Fas-associated phosphatase-1 (FAP-1) was equally expressed in these three samples (data not shown).

Increased Serum Levels of sFas

Both ALL and AML cells expressed the Fas mRNA variants mostly characterized by a deletion of the transmembrane exon 6 domain. These results encouraged us to measure serum levels of sFas in leukemia patients using ELISA. Serum samples were available from 3 patients with ALL and 4 patients with AML. sFas levels were significantly elevated in 5 of 7 cases at onset (Fig. 3). There was no apparent correlation between FasDel6

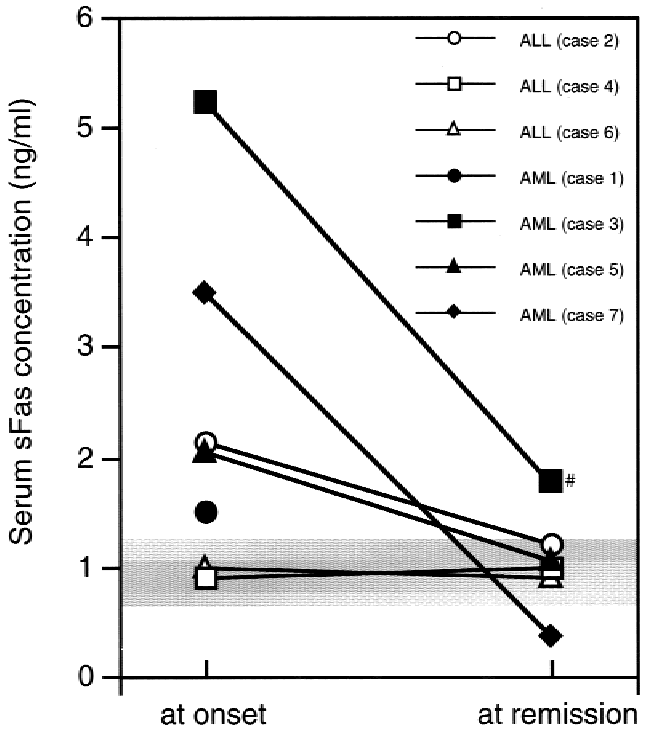

Fig. 3. Changes of serum sFas concentration from onset to complete remission in patients with ALL (cases 2, 4, and 6) and AML (cases 1, 3, 5, and 7). sFas was measured by ELISA. Gray area represents normal sFas range (0.93 ± 0.3 ng/ml) in normal controls. #: serum sample obtained from a nonresponsive AML patient (case 3) after unsuccessful induction chemotherapy. The percentage of blast cells in bone marrow was 52% as assessed by May–Giemsa morphology.

mRNA expression and serum sFas levels at onset. In addition, sFas levels were measured in 5 patients after complete remission. The values in remission were within normal range in all samples. It is of interest that the elevated level of serum sFas protein as well as the increased percentage (52%) of blast cells in bone marrow was demonstrated in a nonresponsive AML patient (case 3) after unsuccessful induction chemotherapy.

DISCUSSION

The growth and death of leukemia cells are modulated by external signals that stimulate apoptosis via cell surface molecules. The Fas receptor has now emerged as an important cellular component that mediates apoptotic cell death [1–3]. Fas was shown to be expressed on the majority of leukemia cells. However, expression of cell-surface Fas receptor, although requisite for the induction of Fas-mediated cell death, does not always predict the biological function of this protein. The failure to trigger Fas-mediated cell death in insensitive cells may involve both pre-receptorial and post-receptorial mechanisms other than those related to the presence of Fas on cell surface. In the present study, the expression of Fas mRNA variants, which code for the sFas proteins lacking the transmembrane domain, was investigated using the RT-PCR technique. The sFas protein lacking the transmembrane domain of full-length Fas results from an alternate mRNA splicing event rather than proteolytic cleavage [20] and may antagonize both anti-Fas MoAb and FasL killing. The biological role of sFas involves binding of FasL before interaction with cell-surface Fas receptor. Alternatively, sFas is able to form trimer complexes with cell-surface Fas to interfere with effective signal transduction [21]. Since FasL expressed on natural killer cells and T lymphocytes is a critical component of cytotoxic effector function, overexpression of sFas might protect leukemia cells from host immunosurveillance and apoptosis.

We found that both ALL and AML cells expressed a distinct RT-PCR product named FasDel6 in addition to the expected fragment (FasFull) corresponding to the full-length Fas cDNA. This product was approximately 60 bp smaller than the FasFull fragment. Furthermore, PCR-Southern blot analysis revealed that the oligonucleotide probe specific for exon 6 coding for the transmembrane domain did not react to the FasDel6 fragment. Although the nucleotide sequence was not determined, FasDel6 fragment is most likely to be a Fas mRNA variant lacking the transmembrane domain, previously called FasExo6Del [21]. This most predominant variant is characterized by a deletion of 63 bp, starting at nucleotide position 700 and ending at position 762 of the full-length cDNA. This in frame deletion results in a cDNA encoding a Fas protein lacking the last five amino acid residues of the extracellular domain and 16 of the 17 amino acids of the transmembrane domain. In addition to the FasDel6 fragment, four fainter and smaller mRNA products were observed, although they were not precisely analyzed in the present study. Papoff et al. described five Fas mRNA variants derived from alternative splicing of the primary transcript and coded for soluble Fas proteins [21]. Importantly, all soluble proteins were shown to block apoptosis induced by either an agonistic MoAb or by natural FasL in Fas-sensitive cells.

Expression of Fas mRNA variants in leukemia cells among individual ALL and AML patients varied. There was no apparent correlation between expression of FasDel6 variant and sensitivity to Fas-mediated growth inhibition. However, it should be noted that, in a 6-year-old boy with AML (case 1) at the first BM relapse, there was an increase in the FasDel6 cDNA concomitantly with a decrease in the expression of the FasFull transcript. Leukemia blasts at the first relapse became completely resistant to Fas-mediated growth inhibition. In contrast, blast cells at onset and the second relapse expressed relatively smaller amounts of FasDel6 mRNA and were sen-

sitive to Fas-mediated growth inhibition. It has been suggested that the generation of Fas and Fas variant transcripts could be tightly inter-regulated, since both of them are derived from the same precursor mRNA [29]. Nevertheless, the clonal revolution of leukemia blasts at the time of relapse might be associated with the alteration of variant Fas mRNA expression. We assumed that up-regulation of Fas variant transcript coding for sFas proteins might possibly render leukemia blasts resistant to Fas-mediated growth inhibition in certain cases. Apparently, it is necessary to further investigate the alteration of variant Fas mRNA expression correlated with the sensitivity to Fas-mediated growth inhibition in more additional cases.

Besides, since detection of mRNA does not always correlate with expression of the relevant protein, serum levels of sFas protein were measured using ELISA. We found that serum levels of sFas were elevated in certain cases of leukemia at the time of diagnosis and subsequently returned to normal levels after complete remission was successfully achieved. Although more samples should be characterized to determine whether sFas levels in patients' sera are indeed typically elevated at diagnosis and reduced at remission, the presented results were quite consistent with those in the previous reports evaluating quite a number of samples collected from patients with various malignancies [22,23,29]. The serum levels of sFas changed depending on leukemia cell burden, i.e., elevated at onset and decreased in remission. These findings suggest that sFas may possibly originate from leukemia cells. It is worthwhile to investigate whether leukemia cells secrete sFas during in vitro proliferation assays, because residual dormant leukemia cells in samples collected at remission might be incapable of releasing sFas protein. We preliminarily measured sFas levels in culture supernatants of AML and ALL cells to confirm that leukemia cells release sFas into extracellular milieu but could not show the presence of sFas in unconcentrated supernatants. Since sFas was clearly detected in 100-fold concentrated supernatants of leukemia cell lines, but not in unconcentrated supernatants [22], it would be interesting to measure sFas levels in concentrated spent medium of primary leukemia cells, especially AML cells cultured with growth-promoting cytokines, such as IL-3 and GM-CSF. In addition, the serum levels of sFas in our patients were found to be comparatively low (range 0.35–5.25 ng/ml), though the previous reports consistently demonstrated that peripheral blood lymphocytes and B- and T-Cell leukemia cells could release a relatively small amount of sFas into culture supernatants [21,22]. It has been demonstrated that a significant inhibition of apoptotic cell death was observed in Fas-sensitive target cells, when cells were cultured with recombinant sFas to achieve concentrations as low as 20ng/ml [21]. Therefore, sFas concentrations in patients' sera might not be so high as to block Fas-mediated killing of leukemia cells. However, it is postulated that sFas released from leukemia cells could function in paracellular milieu, where higher concentrations of sFas might be attainable. In the present study, we demonstrated that variant Fas mRNA coding for sFas proteins was expressed in leukemia cells obtained from patients at onset, although there was no apparent correlation between FasFull/FasDel6 ratio and the sensitivity to Fas-mediated growth inhibition. The number of samples assayed may be too small to convincingly support the hypothesis that sFas expression protects leukemia cells from Fas-mediated killing. More interestingly, serial sampling experiments in a 6-year-old boy with AML suggested that the alteration of variant Fas mRNA expression could be induced at the time of leukemia relapse and could be correlated with the cellular susceptibility to Fas-mediated growth inhibition. Further studies should be undertaken to confirm that up-regulation of sFas expression could alter the sensitivity to Fas-mediated cell death in a large number of patients. Moreover, it is also interesting to determine the place where sFas released from leukemia cells is functioning. Fas-mediated cytotoxic activity of T and NK cells may contribute to the immunological elimination of leukemia cells [10–12] and provide an alternative strategy for the treatment of leukemia. Inhibiting sFas expression could increase leukemia cell susceptibility to Tl- and NK-cell-mediated cytotoxicity. It is important to understand the molecular controls favoring the generation of sFas and membrane Fas receptor.

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